10. The STE solution used to render the bacterial periplasm hypertonic typically contains little rFab after the bacteria are pelleted. However, rFab leakage may occur if the bacteria are incubated in STE for more than 1 h. Therefore, do not incubate the bacteria in STE for more than 45 min on ice.
11. After centrifugation, care should be taken not to pour the cells out of the bottle because the pellet is loose after incubation in STE. It may be necessary to respin the bottles to form appropriate conditions for good pellet formation.
12. By comparing Western blotting results of crude rFab periplasmic extracts, we observed that the total amount of rFab reactive to anti-mouse Fab Ab was not always detected by the Ni-NTA conjugate. This suggests that the His tag fused to the HC can be proteolytically cleaved within the bacterial periplasm. Therefore, the successful purification of rFabs should be checked after metal chelate chromatography and before further purification. The addition of protease inhibitors to the periplasmic extract may help reduce proteolysis.
13. Concentrated rFab preparations may be subjected to size-exclusion chromatography to remove residual impurities, such as aggregated rFabs and contaminating *E. coli* proteins, which can co-elute from the His-bind column. This may increase the avidity of the rFab preparation.

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Expression of Antibody Fragments in *Pichia pastoris*

Philipp Holliger

1. Introduction

Since the advent of hybridoma technology 25 years ago, monoclonal antibodies (Abs) have revolutionized many aspects of biological research and health care. After some initial setbacks, Abs are also beginning to make an impact as therapeutic agents in the clinic *(1)*. In the last decade, novel selection technologies, such as phage display and ribosome display, have emerged, allowing the isolation of Abs directly from diverse repertoires of V genes *(2)*. Phage display, in particular, has become a mature technology, allowing Abs with nanomolar (or even subnanomolar) affinities to be made to order against virtually any Ag, including self Ags (*3*; *see* **Note 1**). Furthermore, using high-throughput technologies, such as robotics and array screening, a multitude of Abs against a given Ag (or mixtures thereof) can now be isolated simultaneously, greatly increasing the options for assay or drug development (*see* **Note 2**).

Regardless of the method of isolation, Abs have to be expressed in recombinant form for screening, characterization, and application. Although both whole Abs and Ab fragments (Fabs *[4]*, Fvs *[5]*, scFvs *[6]*, and diabodies *[7]*) can be expressed in eukaryotic cells (e.g., mammalian yeast, plant, and insect cells *[8]*), it is time-consuming and cost-intensive. Expression in bacteria, particularly secretion to the bacterial periplasm (*see* **Note 3**), is a quick and cheap alternative and is best-suited for the screening and characterization of a large number of Ab variants. Some phagemid vectors *(9)* even offer a built-in switch between phage display and soluble expression, allowing direct screening of Ab fragments isolated by phage selection without the need for

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EXHIBIT A

recloning. However, because of the lack of glycosylation, only Ab fragments and not whole Abs (*see* above) can be produced in a functional form in bacteria.

Expression yields in bacteria can vary widely between different Ab fragments, but yields of 1–10 mg/L are typical for shaker-flask cultures. Using fermentation technology, expression levels of up to 1 g/L can be reached ***(10)***. Generally, expression yields are a function of the Ab fragment sequence and format (e.g., Fv vs Fab), rather than the expression system. Fvs (5–50 mg/L) often give the highest expression yields (but are sometimes unstable), followed by scFvs, then diabodies, with Fabs usually giving the lowest expression yields (0.1–1 mg/L). As a rule of thumb, Ab fragments derived from phage libraries tend to give higher yields than those recloned from hybridomas. However, some Ab fragments are generally difficult to express in *Escherichia coli*. Although yields of difficult fragments can sometimes be improved through protein engineering ***(11,12)*** or selection ***(12)***, no general rules have emerged.

A pragmatic alternative to time-intensive optimization of bacterial expression is the use of a eukaryotic expression host. The methylotrophic yeast, *Pichia pastoris*, combines some of the advantages of eukaryotic expression systems, e.g., more efficient folding of multidomain and cys-rich proteins, with the speed and cost efficiency approaching that of prokaryotic systems ***(13)***. Optimal expression in *Pichia* is dependent on a range of factors, including codon usage ***(14)***, aeration, temperature control (at 28–30°C: *Pichia* is temperature-sensitive) and methanol (MeOH) concentration (when using the alcohol oxidase 1 [AOX1] promoter). Protease-sensitive proteins are usually not well expressed, because *Pichia* secretes a number of proteases. Nevertheless, *Pichia* has become a popular host for heterologous protein expression ***(13)***, and a range of Ab fragments, including scFvs and diabodies, have been successfully expressed in *Pichia*, with yields up to 200 mg/L ***(15)*** in shaker flasks and >1 g/L in fermentor cultures.

This chapter focuses on the expression of functional Ab fragments by the yeast, *P. pastoris* ***(3)***. Using appropriate expression vectors, the Abs are secreted into the yeast culture supernatant, and purified using affinity chromatography. The Ag specificity and binding affinity of the Abs can be determined using BIAcore technology or other suitable methods.

2. Materials

1. *P. pastoris* strain, GS115 (Invitrogen) (*see* Note 4).
2. YP medium: 1% (w/v) yeast extract, 2% peptone.
3. YPD medium: 1% yeast extract, 2% peptone, 2% glucose.
4. YPDS medium: 1% yeast extract, 2% peptone, 2% glucose, 1 *M* sorbitol. For YPDS plates, add 2% (w/v) agar.

5. Sterile Millipore H_2O.
6. 1 *M* sorbitol (SORB).
7. *Pichia* expression vectors: pPIC (AOX promoter) or pGAPZ (Invitrogen) (*see* Note 4). Both vectors have a C-terminal c-myc epitope tag for convenient immunodetection with an anti-myc Ab (9E10) (Invitrogen), as well as a C-terminal hexahistidine tag for immobilized metal-affinity chromatography (IMAC) purification.
8. Ab clone. Because bicistronic expression works only poorly in *Pichia* (unlike *Escherichia coli*), it is preferable to use single-chain Ab formats (e.g., scFv, diabody). Two-chain Ab formats (e.g., Fvs, Fabs, bispecific diabodies) require that the two chains be cloned and transformed separately.
9. Appropriate restriction enzymes and DNA purification and other reagents for molecular cloning of Ab sequences.
10. *E. coli* strain for propagation of plasmid vectors, e.g., TG1.
11. Zeocin (Invitrogen): stock solution 100 mg/mL. Store at –20°C (in the dark).
12. 2TY medium, supplemented with 0.1–5% (w/v) glucose. Autoclave for sterilization, then supplement with sterile-filtered (0.2 µm) glucose (20%).
13. TYE agar (for plates), supplemented with 0.1–5% (w/v) glucose. Autoclave, then supplement with sterile-filtered glucose (20%).
14. TE: 10 m*M* Tris-HCl, 1 m*M* ethylyenediamine tetraacetic acid (EDTA), pH 8.0. Filter-sterilize.
15. Electroporator, e.g., Bio-Rad Genepulser.
16. Methanol.
17. 1 *M* Phosphate buffer: 132 mL 1 *M* KH_2HPO_4, 868 mL 1 *M* KH_2PO_4. Adjust pH to 6.0 with KOH. Filter-sterilize.
18. 10X YNB: 134 g yeast nitrogen base (with NH_4SO_4)/L MilliQ H_2O. Autoclave.
19. 500X B: 20 mg biotin/100 mL MilliQ H_2O. Filter-sterilize.
20. 10X GY: 10% glycerol (v/v) in MilliQ H_2O. Filter-sterilize.
21. BMGY: 100 mL 1 *M* phosphate buffer, pH 6.0, 100 mL 10X YNB, 2 mL 500X B, 100 mL 10X GY in 1 L of YP medium. Filter-sterilize.
22. BMMY: as BMGY, but replace the 10X GY with 100 mL 5% MeOH (v/v) in H_2O. Filter-sterilize.
23. BIAcore machine and software, CM5 BIAcore chip.
24. N-ethyl-Ni-(diaminopropyl) carbodimide (EDC); (*N*-hydroxysuccimide (NHS).
25. Ag of interest, purified.
26. 100 m*M* Na acetate, pH 6.0–4.0; 1 *M* ethanolamine.
27. Phosphate buffered saline (PBS).
28. Ni-NTA resin (Qiagen).
29. IMAC phosphate buffer: 29.82 g NaH_2PO_4, 5.52 g $NaH_2PO_4 \cdot H_2O$, 147 g NaCl/L. Adjust the pH to 7.5 with 1 *M* NaOH.
30. Imidazole (Sigma).
31. IMAC Loading buffer: 50 mM IMAC phosphate buffer, pH 7.5, 0.5 *M* NaCl, 20 m*M* imidazole. Dilute IMAC phosphate buffer fivefold in H_2O, then add imidazole powder to give a final concentration of 20 m*M*. Store at 4°C.

3. Methods

3.1. Preparation of Electrocompetent Pichia GS115

1. Inoculate a single colony of *Pichia* GS115 into 5 mL YPD medium and grow overnight at 30°C.
2. Dilute the overnight culture 1:1000 into fresh YPD medium (e.g., add 1 mL overnight culture into 1 L) and grow overnight at 30°C.
3. Pellet the cells at 1500*g* for 20 min at 4°C, then resuspend in an equal volume of ice-cold sterile Millipore H_2O.
4. Pellet the cells, then resuspend in 0.5 vol ice-cold Millipore H_2O.
5. Pellet the cells, then resuspend in 0.2 vol ice-cold sterile 1 *M* SORB.
6. Pellet the cells, then resuspend in 0.005 vol ice-cold sterile 1 *M* SORB.
7. Use the cells for transformation, or store in 0.1 mL aliquots by flash-freezing on dry ice and store at –70°C (*see* Note 5).

3.2. Cloning of Ab Fragments for Expression in P. pastoris

1. Clone the selected Ab fragment(s) into the appropriate *Pichia* expression vector in *E. coli* using standard cloning procedures (*see* Notes 6 and 7).
2. Prepare plasmid DNA from the resulting clones by miniprep procedures, then linearize with *Avr*II (pGAPZα) or *Bst*XI (pPICZ). Extract the digests with phenol:chloroform (1:1) once, and precipitate the DNA with ethanol. Resuspend the precipitated pellet in 5 µL TE.
3. Add 2.5 µL DNA to 50 µL electrocompetent *Pichia* cells and electroporate at 1.5 kV, 25 µF, and 200 Ω. Resuspend the cells in 1 mL 1 *M* SORB and incubate for 2 h at 30°C.
4. Plate the transformed cells on YPDS plates containing 50 µg/mL zeocin and incubate at 30°C. Colonies (10–1000) will appear in 3–4 d.

3.3. Expression of Ab Fragments in Pichia (see Note 8)

3.3.1. Expression in pPIC (MeOH Induction) (see Note 9)

1. Inoculate a colony expressing a pPIC/Ab clone into 1 mL YPD medium containing 0.1mg/mL zeocin and grow overnight at 30°C.
2. Dilute the overnight culture 1:100 into fresh YP medium (e.g., dilute 0.1 mL into 10 mL) and grow for 24 h at 30°C. Add MeOH to a final concentration of 0.5% (v/v) and grow for a further 24 h. Repeat the MeOH addition every 24 h until the culture is harvested after 1–4 d (*see* Note 10).

   Alternatively, dilute the overnight culture 1:100 into fresh BMGY medium (e.g., dilute 0.1 mL into 10 mL) and grow at 30°C to an optical density 600 nm of 4.0. Pellet the cells by centrifugation at 1500*g* for 20 min, then resuspend in an equal volume of BMMY medium and grow for 24 h at 30°C. Add MeOH (0.5% [v/v] final concentration) and grow for a further 24 h. Repeat the MeOH addition every 24 h until the culture is harvested (after 1–4 d).
3. Spin the culture at 10,000*g* for 30 min at 4°C and collect the supernatant (*see* Note 11). The supernatant can be used directly for analysis of Ab expression

(e.g. by enzyme-linked immunosorbant assay [ELISA] or BIAcore) or can be stored and/or purified before use (*see* Notes 12 and 13).

3.3.2. Expression in pGAPZ (Constitutive Expression)

1. Inoculate a colony expressing a pGAPZα/Ab clone into 1 mL YPD medium containing 0.1 mg/mL zeocin and grow overnight at 30°C.
2. Dilute the overnight culture 1:100 (e.g., dilute 0.1 mL into 10 mL) into fresh YPD medium (without zeocin) (*see* Note 14). Grow the culture at 30°C for 1–4 d (*see* Note 11).
3. Harvest the culture supernatant and store or purify the Ab as described in Subheading 3.3.1.

3.4. Analysis of Ab Binding by BIAcore (see Note 15)

This procedure can be used to quickly investigate Ab specificity using crude extracts of yeast culture supernatant as an alternative to ELISA (*see* Note 16). If purified material is used, the method can also be used to determine affinity. More information about the BIAcore instrument and the method can be found at the BIAcore website: http://www.biacore.com.

1. Dock a research-grade CM5 chip (BIAcore) in the BIAcore machine, according to the manufacturer's instructions.
2. Amine-couple 500–5000 resonance units (RU) of the desired Ag, according to the manufacturer's instructions (the amount of Ag this corresponds to depends on its molecular weight, because the BIAcore signal [RU] is mass-dependent). Briefly, activate the chip surface with EDC–NHS (typical injection is 30 µL at 10 µL/min flow rate). Inject the Ag (typically, 100 µg/mL in 100 m*M* Na acetate, pH 6.0–4.0) (*see* Note 17). Stop the coupling reaction by injecting 1.0 *M* ethanolamine, which blocks the remaining activated sites.
3. Filter the recombinant Ab samples through a 0.2 µm filter before injection.
4. Pass the Ab solution over the chip surface (typical injection times range from 1 to 10 min at flow rates of 5–50 µL/min). An increase in RU indicates binding.
5. Plot a graph of RU vs time. Analyze the binding affinity and/or kinetics using the BIAcore software.

3.5. Purification of Recombinant Ab Fragments by IMAC

Like Ab fragments expressed from polyhistidine-tagged *E. coli* expression vectors, Abs expressed in *P. pastoris* using the pPIC or pGAPZ plasmids can be purified by IMAC. The Ab-containing culture supernatants must first be dialyzed against PBS before purification to remove chelating compounds present in the growth media (*see* Note 18).

1. Dialyze the culture supernatant against two changes of PBS (ideally at 4°C). For smaller volumes, dialysis tubing with a 10 kDa cutoff is suitable. For large

volumes, dialysis is best performed using tangential flow filtration using repeated addition of PBS during the concentration process (*see* Note 12).

2. Add the appropriate amount of Ni-NTA resin to an appropriate column and equilibrate the resin with 10 column volume loading buffer (e.g., for 5 mL resin, use 50 mL buffer). 1 mL Ni-NTA resin is usually sufficient to purify 2–3 mg Ab fragment (*see* Note 19).
3. Load the dialyzed Ab preparation onto the column (either by gravity flow or using a peristaltic pump) and collect the unbound fraction.
4. Wash the column with at least 10 column volume loading buffer. If the washing process can be observed using an UV-flowcell, washing should continue until a stable baseline is reached.
5. Elute the Ab fragments using an imidazole gradient from 35 to 200 m*M* in loading buffer (*see* Note 20). Elution peak fractions should ideally be detected using an UV-flowcell. The elution of Ab should be confirmed by ELISA or BCA protein assay (Pierce).
6. Dialyze the Ab fractions into the desired buffer (e.g., PBS) to remove the imidazole, then concentrate the Ab by ultrafiltration using a stirred cell device with an appropriate cutoff (10 kDa for scFvs, 30 kDa for Fabs, and diabodies).
7. Aliquot the Ab preparations for storage. Concentrated Ab preparations (>0.5 mg/mL) in PBS are suitable for freezing. As a rule, preparations should always be flash-frozen in dry ice or liquid nitrogen and never in a –20°C freezer. Once frozen, a –20°C freezer is suitable for short- to medium-term storage.

4. Notes

1. Protocols for the selection of Ab specificities from phage libraries have been published *(16)* and several libraries are available to researchers (http://www.mrc-cpe.cam.ac.uk/phage/index.html).
2. An alternative method for isolating human Abs is transgenic "human" mice with partial human heavy- and light-chain loci inserted into their genomes *(17)*. A possible advantage of this approach may be the ability to use the isolated hybridomas directly for production of whole Abs with no need for further genetic manipulation.
3. Ab fragments can be expressed both intra- and extracellularly, i.e., secreted. Intracellular expression of Ab fragments in *E. coli* usually gives rise to insoluble aggregates (inclusion bodies) that have to be refolded. Secretion from bacteria (to the periplasm) or yeast mimics the natural expression and folding pathway of Abs and often provides a more direct route to functional Ab fragments.
4. *P. pastoris* strains and expression vectors are commercially available from Invitrogen. *Pichia* protocols are available to download from the Invitrogen website (http://www.invitrogen.com/manuals.html).
5. Freezing reduces competence. In order to obtain the highest possible transformation efficiencies, it is advisable to use freshly prepared cells. However, frozen competent cells are perfectly adequate for standard transformations. Before use, thawed frozen cells should be washed once in 0.5 mL ice-cold sterile 1 *M* sorbitol.
6. Ab fragments can be cloned using PCR directly from hybridomas using standard methodology (a kit comprising mouse V-gene-specific primers is available from Pharmacia) or isolated from phage selected from libraries using panning procedures.
7. When using zeocin selection in combination with high-salt media (TYE, 2TY), it is advisable to use a final concentration of 100 µg/mL zeocin for selection. Transformed *E. coli* cells should be incubated for 1–2 h in 2TY, 1% glucose at 37°C, before plating on zeocin plates because zeocin resistance is expressed slowly.
8. There are two types of promoter systems available in *Pichia*; the MeOH-inducible AOX1 promoter and the constitutive glyceraldehyde-phosphate dehydrogenase (GAPDH) promoter. Expression of some proteins can be higher under control of the GAPDH promoter (using glucose as a carbon source) than by MeOH induction of the AOX1 promoter. Both promoters should be tried because expression yields can differ dramatically. Furthermore, expression levels usually vary a great deal among different *Pichia* clones. It is advisable to screen a number of colonies for expression in order to identify high-expressing "jackpot" clones. *Pichia* expression can also depend on good aeration so expression cultures should be grown with vigorous shaking (350 rpm).
9. For optimal protein yields with MeOH induction, the alternative may be more effective than the primary methods.
10. Protein expression takes place over 1–4 d at 30°C. Maximum yields usually are obtained by harvesting on d 2 or 3.
11. Respin the culture if the supernatant is not clear.
12. The cleared supernatant can be used directly in ELISA or BIAcore analysis, or can be stored at –20°C prior to purification. For large-scale preparations (>1 L), it may be advantageous to concentrate the supernatant before purification. Various concentration methods are available (e.g., ammonium sulphate precipitation), but ultrafiltration is preferable. Filter the supernatant through a 16 µm tangential-flow filter (Flowgen Minisette system) with the use of a peristaltic pump to remove small debris. Concentrate the supernatant using the Minisette system, using a tangential-flow filter minisette with an appropriate cutoff (e.g., 10 kDa for scFvs and Fvs, or 30 kDa cutoff for Fabs and diabodies). The concentrate (typically, 0.3–0.5 L) can be stored at –20°C prior to purification.
13. Ab fragments produced in *Pichia* often have nonhomogenous N-termini because of incomplete processing of the leader peptide, giving rise to fuzzy bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. These N-terminal extensions can be shaved off using 5 µg/mL trypsin digestion for 5 min (immobilized TPCK trypsin [Pierce]). The reaction is stopped by addition of Pefabloc trypsin inhibitor (at 5 µg/mL) and removal of the enzyme gel by centrifugation. Because trypsin may also cleave off polyhistidine tags, it is advisable to carry out the digestion after purification.

14. Zeocin selection during expression is unnecessary and can reduce the yield of expressed protein.
15. These methods are not limited to Ab expressed in *Pichia* and can also be used for determining Ag specificity (crude periplasmic extracts) and binding affinity (purified Ab) of Ab expressed in *E. coli*.
16. Either ELISA or BIAcore can be used to determine affinity constants of purified Ab fragments. In my opinion, BIAcore is superior to ELISA-based methods, provided attention is paid to the oligomerization state of the Ab fragment. Multimeric fragments (e.g., some scFvs, bivalent diabodies) bind to solid-phase Ags with much-increased affinity (avidity). Failure to take this into account can lead to an overestimation of affinity by several orders of magnitude. On the other hand, multimerization can be helpful in increasing the sensitivity of Ag-binding assays, particularly for Ab fragments with modest affinities for Ag. For methods relating to Ab multimerization (and expression), *see* ref. *18*. BIAcore can also be used to measure Ag-binding kinetics.
17. For optimal coupling efficiencies, the pH should be determined by experimental analysis (knowledge of the isoelectric point value of the Ag is not sufficient). Coupling should be spontaneous. For slow-reacting Ags, it may be appropriate to slow down the flow rate.
18. Purification by IMAC has advantages beyond other purification methods because of its versatility and mild elution conditions. The commonly used rich medium for *Pichia* (YP) expression (and for *E. coli* [2TY or Luria-Bertani broth]) contain metal-chelating compounds, which strip the metal from the IMAC column (the same also applies for periplasmic preparations from *E. coli*-containing EDTA). Metal loss from the IMAC column is easy to spot because the column loses its blue-green color (in the case of Ni^{2+}) and turns white.
19. Ab fragments can give widely differing expression yields, ranging from 1 to 100 mg/L of induced *Pichia* culture. It is thus advisable to determine approximate expression levels before embarking on purification.
20. Most Ab fragments elute between 50 and 100 m*M* imidazole. Diabodies and triabodies, which have two and three hexahistidine tags, respectively, usually elute at higher concentrations (50–200 m*M* imidazole).

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